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Ray White

PI - Signature

7/26/95

Date

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(5) INTRODUCTION

Characterization of the early events in the development of breast cancer is expected to lead to a better understanding of transformation of normal cells to malignancy and may lead to new approaches for the detection of precancerous lesions and new treatment methods. Since germline mutations in two tumor suppressor genes, *p53* and *BRCA1*, are associated with inherited predisposition to breast cancer⁽¹⁻⁵⁾, and losses of heterozygosity (LOH) on 17p and 17q occur frequently in sporadic breast tumors^(6,7), alterations in the *p53* (17p) and *BRCA1* (17q21) genes represent some of the earliest genetic changes known to occur in the development of breast cancer.

To study the effects of inactivating mutations in the *p53* and *BRCA1* genes early in the breast cancer pathway, we will develop genetically defined human mammary epithelial cell (HMEC) lines by introducing heterozygous and homozygous mutations of each gene using homologous recombination^(8,9). Additionally, we will construct HMEC lines deficient in *p53* protein by expressing the E6 gene of human papillomavirus type 16 (HPV16), which increases the rate of degradation of the *p53* protein⁽¹⁰⁻¹²⁾. The consequences of these genetic changes for cell metabolism will be discovered through controlled *in vitro* comparisons between genetically altered derivatives and their isogenic parent cells. One level of comparison will involve observations of their growth properties, expression of certain cell lineage markers (e.g. keratins, integrins), morphology and behavior. At another level, we will take a global approach to the discovery of metabolic changes associated with genetic alterations in early tumorigenesis by constructing subtraction cDNA libraries⁽¹³⁾ and by differential display⁽¹⁴⁾ to reveal changes in mRNA transcription that are associated with loss of activity of each of these genes. Clones showing differential expression will be sequenced in our Sequence Core Facility at the University of Utah to reveal genes potentially critical in growth control, by reference to databanks.

(6) BODY

The following progress was made during the first year of the funded project (July 1, 1994 to June 30, 1995).

Culture of human mammary epithelial cells (HMEC) *in vitro*

Most breast cancers arise from epithelial cells of the mammary gland. Two types of epithelial cells are most common in the mammary gland: a continuous layer of glandular cells (luminal cells) lines the duct, whereas a discontinuous layer of cells near the basement membrane (basal cells) shares certain features of myoepithelial cells⁽¹⁵⁾. Intermediate phenotypes have also been observed⁽¹⁵⁾. Basal cells predominantly express 5 types of keratins (K), K5, K6, K7, K14, and K17, whereas luminal cells predominantly express 3 types, K8, K18, and K19⁽¹⁵⁻¹⁷⁾. Cancer cells in the breast produce mainly K8, K18, and K19⁽¹⁶⁾. Media are available to culture HMEC *in vitro* (17-20). However, we have found that these media support only limited and slow growth of HMEC when the cells are plated on conventional plastic surfaces. We have recently developed a new system of co-culturing HMEC with fibroblasts, a strategy which seems to support better growth of HMEC of both luminal and basal origin (manuscript in preparation). Briefly, HMEC are maintained in DFCI-1⁽¹⁹⁾ or MCDB 170⁽¹⁸⁾ medium on a separate collagen-coated membrane insert arranged over fibroblasts. Using this co-culture system, we have established more than 40 primary HMEC cultures from reduction-mastectomy and breast-biopsy specimens.

Culture of HMEC from women who are known carriers of tumor suppressor gene mutations

Breast biopsies from women who are members of high-risk breast cancer families are being made available to us through interested clinicians at the University of Utah. We have established several primary HMEC cultures from biopsy specimens taken from women who carry abnormal alleles of the *BRCA1*, *APC*, and *NF1*

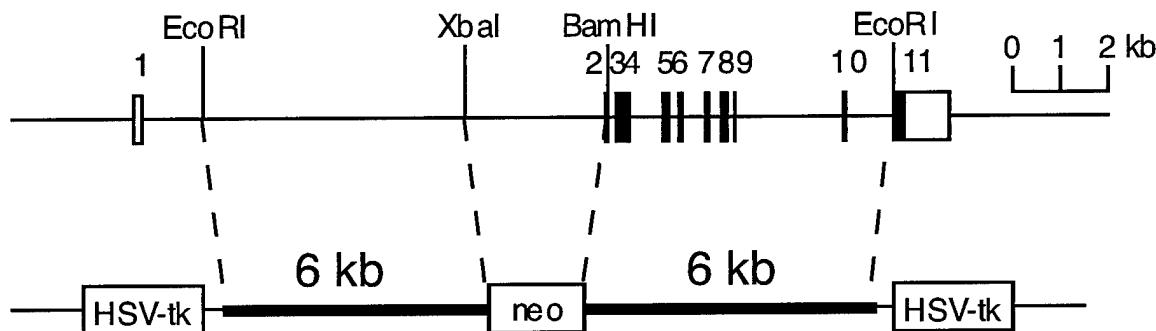
genes. Additionally, we are to receive biopsy specimens from women who carry constitutional mutations in the *p53* gene (Li-Fraumeni syndrome) within 6 months.

These cultures will become important and convenient resources for constructing homozygously mutant HMEC lines, since they already carry one mutated allele.

***p53* gene knock-out by homologous recombination**

We will create *p53* knock-outs via homologous recombination in HMEC. Using the *p53*-containing cosmid clone that was isolated in our laboratory and the replacement vector pKT2(8), we have constructed a *p53* replacement vector which contains three essential components: a positive selectable marker (neo), a negative selectable marker (HSV-tk), and 6 kb each of genomic DNA sequence from both the 5' and 3' regions of the *p53* gene, as shown in Figure 1. The positive selectable marker carries a neomycin-resistance gene under the control of human cytomegalovirus promoter. Upon homologous recombination, *Xba*I/*Bam*HI genomic DNA containing the translation-initiation codon in exon 2 is replaced by the positive selectable marker. Cells that have incorporated the negative selectable marker (HSV-tk) through random integration will be counter-selected by gancyclovir(8). This replacement vector, linearized by digestion with a single restriction enzyme, has been transfected into HMEC with DOTAP liposomal transfection reagent (Boehringer Mannheim). Cells have been selected with medium containing gancyclovir and G418, and we are now in the process of screening for successful integration of vector.

Figure 1



It is essential to develop a derivative line with homozygous mutation. One approach involves sequential targeting by two separate constructs using two different selectable markers (e.g. neomycin and hygromycin)(21). Another approach would be to select for homozygous cells spontaneously produced from heterozygous cells by a variety of mechanisms including gene conversion and chromosomal loss followed by chromosomal duplication. Investigations with other systems have shown that, if the primary integration can be selected with relatively low levels of G418 (100-500mg/ml), cells that have undergone reduction to homozygosity can be selected with high levels of G418 (1-2mg/ml)(22). Fortunately, HMEC are highly sensitive to G418, being effectively killed by 50mg/ml.

In addition to normal HMEC, we intend to use HMEC that already carry constitutional mutations in the *p53* gene (Li-Fraumeni syndrome), where only a single additional disruption is required, when those cells become available.

***BRCA1* gene knock-out by homologous recombination**

From the collection of overlapping P1 clones which were originally isolated in our laboratory from the *BRCA1* locus over the past two years(23), we have identified two P1 clones, 63B3 and 84F2, in which exons 2 to 18 of the *BRCA1* gene are represented. Using these clones as resources for target DNA, we are constructing a *BRCA1* replacement vector similar to the one described above.

Ki-ras gene knock-out by homologous recombination

We have obtained a vector for replacement of the Ki-ras gene which others have used successfully to disrupt the Ki-ras gene in human colon-cancer cell lines⁽²⁴⁾. This construct will serve as a control for our series of homologous recombination experiments.

Retrovirus-mediated gene transfer

The highly efficient retrovirus-mediated gene transfer system⁽²⁵⁾ became available in our laboratory during the past year. This system consists of ecotropic and amphotropic packaging cells and retrovirus vectors that contain varieties of promoters and drug selection markers^(25,26). Using this system, we have successfully introduced various genes into HMEC, including HPV16 E6 and E7⁽²⁷⁾.

Immortalization of HMEC by HPV16 E6 and/or E7 and dominant inactivation of *p53*

For several years, specific interactions between transforming genes of DNA tumor viruses and the cellular regulatory proteins encoded by the *p53* and *RB* genes have been thought to form an important component of transformation by these viruses. It has been found that the DNA virus HPV16, implicated in cervical cancer, divides these interactions between two genes⁽²⁸⁾: the E6 protein increases the rate of degradation of the *p53* protein through the ubiquitin pathway⁽¹⁰⁾, leading to undetectable levels in transfected HMEC lines^(11,12), and the E7 protein apparently binds the *RB* protein. We have successfully immortalized HMEC either by HPV16 E6 alone, E7 alone, or E6 and E7 together *via* retrovirus-mediated gene transfer⁽²⁷⁾. Biochemical analysis has shown an expected loss of *p53* protein in both E6- and E6+E7- immortalized HMEC, but not in E7-immortalized HMEC (data not shown). Immunohistochemical staining of keratins has demonstrated that the majority of E6- immortalized cells exhibit the character of basal cells (K14+, K19-), whereas E7- or E6+E7- immortalized cells exhibit characteristics of luminal (K14-, K19+), basal (K14-, K19+), and mixed (K14+, K19+) cellular origins (data not shown). Recently, Wazer et al.⁽²⁹⁾ reported a similar observation, and they discussed the possibility of different susceptibilities to viral oncogenes among breast epithelial cell subtypes.

These observations are particularly useful for this project because immortalization may be necessary to support construction of HMEC with targeted tumor suppressor genes by homologous recombination. By conditionally expressing these viral genes, we will be able to determine whether immortalization has been achieved by the genetic alterations we are creating.

New strategies for conditional immortalization of HMEC

As described above, although adequate doublings are available with primary HMEC culture for the selection of single-step heterozygous derivatives, these cultures may not survive long enough for the construction and testing of multi-step derivatives. It would be useful to have a defined, conditional immortalized system.

We first tested the hypothesis that the tsA58 or U19tsa allele of SV40 large T antigen can conditionally immortalize HMEC. These temperature-sensitive alleles of SV40 large T antigen have been used as conditionally immortalizing tools in several cell strains and in transgenic mice⁽³⁰⁾. However, in our experience both temperature- sensitive constructs have failed to immortalize HMEC at their permissive temperature, although the wild-type SV40 large T antigen has been reported as an immortalizing tool in HMEC⁽¹⁷⁾.

The tetracycline-responsive promoter system developed by Gossen and Bujard⁽³¹⁾ is in widespread use in many laboratories. In their system, a gene of interest is cloned downstream of the minimal human cytomegalovirus (HCMV) immediate early (IE) promoter fused to seven copies of the *Tn*-10 tetracycline operator (tetO). Expression from this promoter can be tightly controlled in stable cell lines that constitutively express a fusion protein termed tetracycline-controlled transactivator (tTA). The tTA consists of the tetracycline repressor combined with the C-terminal transcriptional transactivation domain of VP6 of herpes

simplex virus. Binding of the tTA cannot occur in the presence of tetracycline, and promoters containing tetO sequences are virtually silent in tTA-expressing HeLa cells. However, following removal of tetracycline, activity of the promoter is induced by up to 5 orders of magnitude. Although this original system has widespread utility, it is not functional in all cell types because the background activity of the tetO/HCMV IE promoter varies significantly in certain cell lines⁽³²⁾. To examine whether this system would be applicable in our HMEC, we first established stable HMEC lines constitutively expressing the tTA by infecting retrovirus containing tTA and a neomycin-resistance gene. Cells were selected in medium containing 50mg/ml of G418, and resistant colonies were amplified as sister cell lines. Two sister lines, HMEC-tTA-1 and HMEC-tTA-2, were transiently transfected with pUHC13-3 plasmid containing the luciferase gene downstream to the tetO/HCMV IE promoter⁽³¹⁾ with DOTAP liposomal transfection reagent (Boehringer Mannheim). Transfected cells were cultured for 48 h in the presence or absence of 1mg/ml tetracycline and assayed for luciferase activity. Results from duplicate experiments (Table 1) showed 1) low basal activity of the luciferase in the presence of tetracycline, and 2) up to 160-fold induction of the luciferase activity in the absence of tetracycline, indicating this conditional expression system is applicable in our HMEC lines.

Table 1

average luciferase activity

		average luciferase activity
HMEC-tTA-1	DNA (-)	52
	Tet (+)	67
	Tet (-)	2432
HMEC-tTA-2	DNA (-)	108
	Tet (+)	187
	Tet (-)	9760

We are now constructing plasmids similar to pUHC13-3 which will contain the HPV16 E6 and/or E7 genes in place of the luciferase gene, and a drug-selection marker (hygro) to select for stable clones. These plasmids will be transfected into HMEC constitutively expressing the tTA and cells will be selected in medium containing 20mg/ml of hygromycin. Stable lines that result will be tested for conditional immortalization as well as conditional inactivation of the *p53* protein.

In addition to the tetracycline system, we are also testing another inducible system, sheep metallothionein promoter. This promoter differs from that of mouse or human in that it does not contain a recognized basal level expression sequence motif; also, it may be unresponsive to glucocorticoids present in HMEC culture media^(33,34). This system has been used successfully to induce transformation of rat a fibroblast cell line by conditionally expressing v-myc oncogene⁽³⁴⁾.

Differential Display

The differential display technique originally described by Liang and Pardee⁽¹⁴⁾ has been applied in several projects in our laboratory with moderate success over the past two years, using the DNA oligonucleotide primers and conditions originally described. Recently, several groups reported significant improvements involving choice of DNA oligonucleotide primers, PCR conditions, and non-radioactive detection systems. Many of these improvements have been incorporated and tested in our laboratory with very promising results. The differences are currently being verified by two independent means; firstly, repeated experiments, where RNAs from different harvests of cells serve as templates for the 1st-strand cDNA synthesis, are being performed to reproduce the original observation. Secondly, following the recovery and reamplification of RT-PCR products from the gels, these products are tested either by Northern blotting or by quantitative RT-PCR to confirm differences in expression level.

(7) CONCLUSIONS

During the past year, the first year of our currently funded grant, we have established a series of primary HMEC cultures from normal individuals and from carriers of various tumor suppressor mutations, using our newly developed co-culture system. Homologous recombination experiments are in progress; one of the targeting vectors has been constructed and introduced, and candidate targeted HMEC clones are now in analysis. Immortalization of HMEC was achieved by introducing HPV16 E6/E7 *via* retrovirus-mediated gene transfer. We have shown promising results for a conditional expression system, which will be applied for the construction of a controlled system to conditionally immortalize HMEC. One of the tools to characterize genetically defined HMEC, differential display, has been tested and is available.

However, in addition to the experiments outlined above, we think that the following experiments are also necessary.

A) Recently, Thompson et al.(35) reported that when *BRCA1* was down-regulated following addition of a *BRCA1* antisense oligonucleotide, they observed accelerated growth of both normal and malignant breast epithelial cells, which seemed to correspond to suppression of *BRCA1* expression. Thompson's group did not see this effect in non-mammary epithelial cells. If we can confirm their observation in our culture system by growing cells in the presence and absence of the same antisense oligonucleotide, these cells will also be characterized.

B) In parallel with our experiments involving homologous recombination and conditional expression of HPV16 E6/E7, we will screen HMEC lines established from women already carrying *p53* or *BRCA1* mutations for the spontaneous loss of the normal allele of each of these genes. Several human cell lines have been shown to produce homozygous loci from originally heterozygous sites at a low frequency, although the mechanism is not defined(36). A recent study indicated that HMEC lines established from a Li-Fraumeni patient showed a high frequency of spontaneous immortalization when the wild-type *p53* allele was inactivated(37), suggesting that once HMEC cultures are established it will not be difficult to obtain homozygosity for mutant alleles. Clones that have undergone reduction to homozygosity will be detected by means of semi-automated, PCR-based fluorescence genotyping at our Genotyping Core Facility at the University of Utah.

C) As an alternative means of inactivating *p53* in HMEC, we will develop cell lines in which the *p53* protein is rendered non-functional by: 1) over-expressing the *mdm-2* oncogene(38), the protein product of which is known to bind and inactivate *p53*; and by 2) over-expressing variants of the *p53* protein which are believed to inhibit the normal function of *p53* by a dominant-negative mechanism(38). These experiments are important because, since *p53* is not the only protein to bind to HPV16 E6, degradation of *p53* may not be the only reason for immortalization and subsequent changes caused by HPV16 E6.

The resulting genetically defined HMEC lines will be important resources for studying some of the early genetic changes that are thought to occur in the development of breast cancer. Characterization of the consequences of such changes will be highly relevant to the understanding of tumor progression and may lead to new applications for early detection, prevention, and treatment of breast cancer.

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(9) APPENDIX

List of salaried personnel:

<u>Name</u>	<u>Position</u>
Nori Matsunami	Research Associate
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